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Deletion of 150 kb in the minimal DiGeorge/velocardiofacial syndrome critical region in mouse

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Deletions or rearrangements of human chromosome 22q11 lead to a variety of related clinical syndromes such as DiGeorge syndrome (DGS) and velocardiofacial syndrome (VCFS). In addition, patients with 22q11 deletions have an increased incidence of schizophrenia and several studies have mapped susceptibility loci for schizophrenia to this region. Human molecular genetic studies have so far failed to identify the crucial genes or disruption mechanisms that result in these disorders. We have used gene targeting in the mouse to delete a defined region within the conserved DGS critical region (DGCR) on mouse chromosome 16 to prospectively investigate the role of the mouse DGCR in 22q11 syndromes. The deletion spans a conserved portion (~150 kb) of the proximal region of the DGCR, containing at least seven genes (*Znf74l*, *Idd*, *Tsk1*, *Tsk2*, *Es2*, *Gscl* and *Ctp*). Mice heterozygous for this deletion display no findings of DGS/VCFS in either inbred or mixed backgrounds. However, heterozygous mice display an increase in prepulse inhibition of the startle response, a manifestation of sensorimotor gating that is reduced in humans with schizophrenia. Homozygous deleted mice die soon after implantation, demonstrating that the deleted region contains genes essential for early post-implantation embryonic development. These results suggest that heterozygous deletion of this portion of the DGCR is sufficient for sensorimotor gating abnormalities, but not sufficient to produce the common features of DGS/VCFS in the mouse.

INTRODUCTION

DiGeorge syndrome (DGS) and velocardiofacial syndrome (VCFS) are part of a group of related human dysmorphic syndromes that result from deletion or rearrangement of chromosome 22q11 (1). These syndromes are characterized by impaired cellular immunity from thymic hypoplasia or aplasia, hypocalcemia due to absence of parathyroid glands, congenital heart defects and typical facial dysmorphisms (2). These phenotypes are thought to result from neural crest developmental abnormalities in the third and fourth pharyngeal pouches (3,4). A reasonable hypothesis is that a single gene or genes within 22q11 may be critical during embryogenesis for normal development, differentiation and migration of neural crest cells in a dosage-sensitive manner. Hence, these syndromes are referred to as the 22q11 haploinsufficiency syndromes.

However, the genetics of these syndromes are more complex than predicted for a typical segmental aneusomy syndrome. The severity of the phenotype does not necessarily correlate with the extent of deletion (5–7) and family members with identical deletions can have widely differing phenotypes (8). Greater than 90% of persons with DGS, VCFS and conotruncal anomaly face syndrome (CAFS) have large interstitial deletions (~2 Mb), apparently mediated by the presence of direct repeats that flank this 2 Mb region (9). Several genes within this DGS critical region (DGCR) are good candidates for playing important roles in DGS and related syndromes. However, molecular analysis of rare patients with no detectable deletions has so far failed to identify a single gene responsible for 22q11 phenotypes and single gene knockouts of several of these candidate genes in the mouse have failed to reveal phenotypic defects similar to those observed in DGS/VCFS (10–12). In the case of DGS, different investigators have defined distinct, non-overlapping 'critical regions' on the

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basis of shortest region of deletion overlap mapping of rare cases with atypical deletions or seemingly balanced translocations (13–16). These results suggest that the notion of 22q11 haploinsufficiency does not adequately describe the complexity of this region in mediating human diseases.

Besides these phenotypes, patients with 22q11 deletions have an increased incidence of schizophrenia and other neuropsychiatric disorders (17–19) and several studies have mapped susceptibility loci for schizophrenia to this region (20–22). These results suggest that there are genes within the DGCR that may participate in human neuropsychiatric disorders.

To attempt to elucidate the genetic basis of DGS/VCFS and other related 22q11 haploinsufficiency syndromes, we prospectively deleted a portion of the DGCR in the mouse on chromosome 16. Using conventional gene targeting in ES cells, we deleted a conserved portion of one of the defined non-overlapping critical regions, the minimal DiGeorge critical region (MDGCR). Boundaries of this region were defined by deletion and translocation breakpoint mapping data from selected patients and genes within this region are thought to be strong candidates for the pathogenesis of DGS/VCFS (23,24). The ~150 kb deletion encompasses seven genes, six of which are in the MDGCR. Heterozygous and homozygous deleted mice were investigated for phenotypes of 22q11 deletion syndromes, including behavioral phenotypes. We determined that this region plays a role in regulating normal sensorimotor gating, a process that is defective in patients with schizophrenia, and that homozygous deleted mice died early in embryogenesis, soon after implantation. However, heterozygous mice displayed no common phenotypes consistent with DGS/VCFS.

RESULTS

Production of mice with heterozygous deletion within the DGCR

Using conventional replacement gene targeting techniques, we produced embryonic stem (ES) cells with the deletion of ~150 kb of the proximal region of the DGCR. This deleted region contains at least seven genes: *Znf74l*, *Idd*, *Tskl*, *Tsk2*, *Es2*, *Gscl* and *Ctp*. A targeting construct was made utilizing the ends of two overlapping BAC clones (Fig. 1A). Fifteen to twenty kilobases of flanking sequence at the distinct ends of these BACs were cloned on either side of PGK*neo* and an HSV *TK* gene was placed on each side of the construct to allow for positive and negative selection. Approximately 1000 ES clones were screened and two clones appeared positive after Southern blot analysis (Fig. 1B). One true homologous recombinant was confirmed by both pulsed field gel electrophoresis (PFGE; Fig. 1C) and fluorescence *in situ* hybridization (FISH; data not shown), using several probes from the deleted region. Healthy and viable male chimeras were generated using the single deleted ES clones and these chimeras were used to produce heterozygous mouse lines in both inbred 129/SvEv-Tac (129S6) and mixed (129S6 × NIH Black Swiss) backgrounds. FISH was used to confirm that the heterozygotes did in fact carry the deletion (Fig. 1D).

Essential role of the deleted region in early embryonic development

Animals heterozygous for the deletion allele were viable, fertile, generally healthy and born in appropriate Mendelian ratios from heterozygous × wild-type (data not shown) or heterozygous crosses (Table 1). Cumulative genotyping of litters from heterozygous crosses between E7.5 and birth demonstrated that the ratios of +/+:+/–:–/– F_2 animals were 28:48:0, with 30 resorptions. The number of resorptions is appropriate to account for the loss of all homozygotes, suggesting that homozygous deletion is compatible with implantation, but embryos degenerate soon thereafter. Between E5.5 and E7.5, individual litters contained normal appearing embryos and empty decidua (data not shown), suggesting that complete deficiency of the deleted region resulted in early post-implantation embryonic lethality. Cumulative genotyping of adult offspring from heterozygous crosses demonstrated that only +/+ and +/– animals survived at a 1:2 ratio (Table 1).

We examined the behavior of blastocysts isolated from heterozygous crosses cultured *in vitro* and blastocyst outgrowths were genotyped by PCR at the end of the culture period. Wild-type (Fig. 2A), heterozygous (data not shown) and homozygous (Fig. 2B) blastocysts attached to the substratum, although all homozygous blastocysts (Fig. 2D) hatched later than wild-type (Fig. 2C) or heterozygous blastocysts. Trophoblast cells from wild-type blastocysts flattened and expanded while the inner cell mass grew on top of the trophoblast cells (Fig. 2C and E). In contrast, the inner cell mass cells of homozygous blastocysts often did not hatch after 4 days (Fig. 2D) and degenerated soon thereafter (Fig. 2F). Trophoblast flattening and expansion was also decreased in all homozygous blastocysts. None of these abnormalities was detected in wild-type or heterozygous blastocysts. Overall, these results demonstrate an essential role for the deleted region in early post-implantation development, perhaps as a consequence of inner cell mass and trophoblast defects.

Increased prepulse inhibition in heterozygous deleted mice

Because of the possible involvement of the DGCR in schizophrenia and since individuals with DGS can have mild forms of mental retardation, we hypothesized that the deleted mice may have impaired sensorimotor gating and impaired learning and memory. Therefore, we evaluated the responses of heterozygous deleted and wild-type mice in a battery of behavioral tests that have been used to assess various domains of central nervous system function (25–27). This battery included the prepulse inhibition paradigm for sensorimotor gating and the conditioned fear test to assay for learning and memory impairments.

The behavioral responses of heterozygous deleted mice were significantly different from wild-type mice on the prepulse inhibition test (Fig. 3B). Overall, the percent prepulse inhibition response in heterozygous mutant mice was abnormal, but in contrast to our hypothesis the percent inhibition was significantly higher in deleted mutants than in wild-type controls ($P = 0.016$). Although the overall effect of genetic background was not significant ($P = 0.349$), there was a significant genetic background–prepulse sound level interaction ($P = 0.0003$). This interaction reflects the fact that mice of the mixed genetic

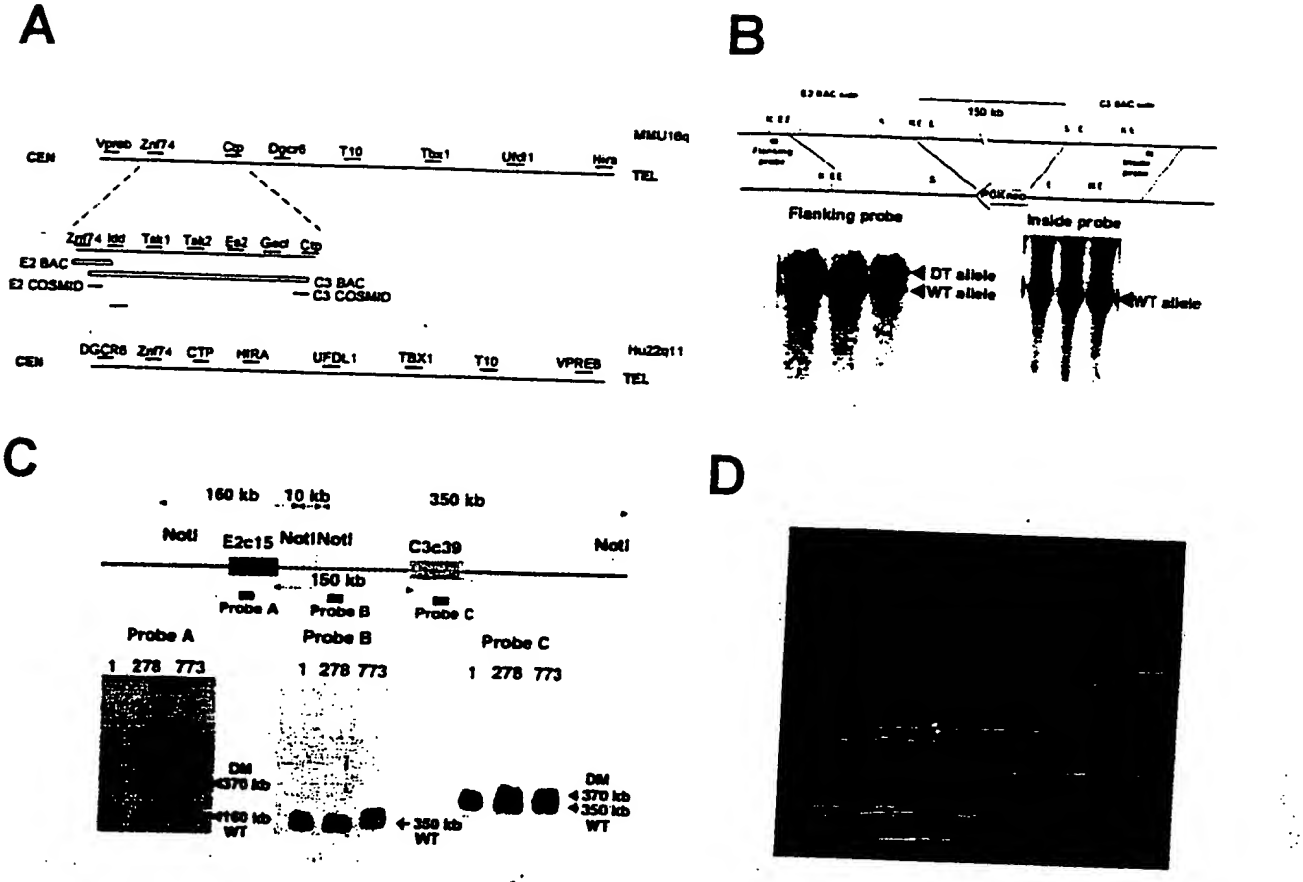

Figure 1. Generation of deleted mice. (A) Schematic representation of the structure of the deleted region showing genes mapped to this region. BAC and cosmid clones from which the targeting vector was derived are shown, along with the position of the probe used for FISH analysis, and the extent of the deletion is denoted by a dashed line. (B) Southern analysis of deleted ES clones. (Top) Schematic representation of the locus and targeted allele, replacing the 150 kb region with PGK*neo*. Each arm of the targeting construct was 15–20 kb in length and the locations of the ends of the arms are indicated by the lines connecting the wild-type (top) to targeted (bottom) allele. The positions of the flanking and inside probes are indicated, as are the positions of the restriction sites for *Kpn*I and *Eco*RI. (Bottom) Two potentially targeted clones were detected using *Kpn*I digestion with the flanking probe. The wild-type (WT) allele is ~25 kb, while the deleted (DT) allele is ~30 kb. Using the internal probe, no abnormal bands were detected, suggesting proper homologous recombination. (C) PFGE of the potentially targeted clones. (Top) Schematic representation of the wild-type locus, with the positions of three probes (A, B and C) and locations of the BAC end clones and *Not*I sites. (Bottom) Southern analysis after PFGE, probing with each of the three probes. Clones 278 and 773 each displayed the appropriate bands for correctly targeted clones. (D) FISH analysis of mice with heterozygous deletion. Metaphase spread hybridized with a mouse chromosome 16-specific paint (red) and genomic clone 8.1 (yellow) which is absent from the deleted allele.

Table 1. Genotypes of offspring from heterozygous crosses

	+/+	+/−	−/−	Resorptions
Blastocysts	3	33	4	N/A
Embryos	28	48	0	30
Adult	61	123	0	N/A

background had significantly lower levels of prepulse inhibition compared with mice of the 129S6 inbred background at the two highest prepulse sound levels ($P < 0.029$). No other interactions were significant ($P > 0.317$).

We have not examined the responses of female mice in the inbred background. However, when we performed an analysis of the prepulse inhibition data for male and female mice of the mixed genetic background we also found that overall the deleted mutant mice had significantly higher levels of PPI compared with wild-type mice ($P = 0.048$). There was no overall difference between male and female mice, and the genotype–gender interaction was not significant ($P > 0.27$). These findings support the interpretation that the heterozygous deleted mutant mice have higher levels of prepulse inhibition that do not depend on genetic background or gender.

The acoustic startle response (Fig. 3A) was not significantly different between the two genotypes of male mice ($P = 0.478$) and the difference in startle response between the two genetic backgrounds was not significant ($P = 0.079$) nor was the genotype–genetic background significant ($P = 0.924$).

The performance of heterozygous mutant male mice on the rotarod was also significantly worse than that of wild-type male mice, but this difference was dependent on genetic background. Although the overall effect of genotype was not significant ($P = 0.412$), the effect of genetic background (wild-type versus inbred $P = 0.00015$) and the genotype–genetic background interaction ($P = 0.047$) were statistically significant. *Post hoc* comparison of the genotype–genetic background interaction showed that heterozygous mutants were worse than wild-type mice, but the difference was only significant in mice from the mixed genetic background ($P = 0.049$), not in the 129S6 inbred

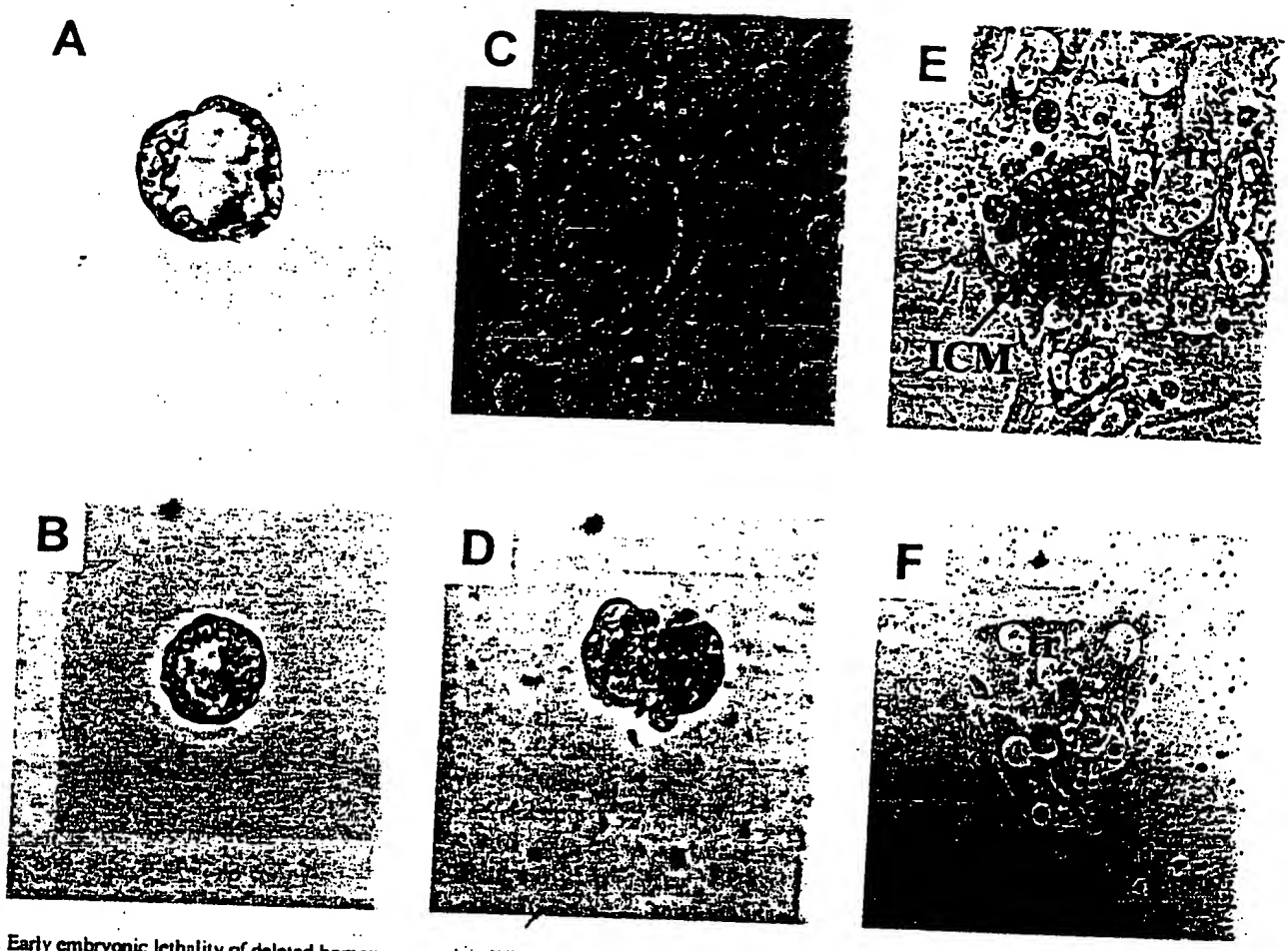

Figure 2. Early embryonic lethality of deleted homozygous mice. Wild-type (A) and homozygous (B) blastocysts were isolated from a heterozygous cross, placed in culture and photographed daily. Photographs of the same wild-type blastocyst at 4 (C) and 6 (E) days in culture and the same homozygous blastocyst at 4 (D) and 6 (F) days in culture are shown. ICM, inner cell mass; tr, trophoblast cells.

background ($P = 0.388$). In addition, the *post hoc* analysis revealed that wild-type mice from the mixed genetic background were significantly better than the 129S6 inbred wild-type mice ($P = 0.0006$), but there were no significant differences between mutant mice from the two genetic backgrounds ($P = 0.115$).

On the remaining tests there were several overall differences in performance between the mixed and 129S6 inbred mice (e.g. total distance traveled in the open field, rearing responses in the open field, the center distance ratio in the open field, light–dark transitions and conditioned fear), but there were no other significant differences between heterozygous deleted and wild-type mice and there were no significant genotype–genetic background interactions. Thus, the overall behavioral responses of heterozygous mice were similar ($P > 0.05$) to those of wild-type mice on the neurological screen, open field, light–dark box, habituation of the acoustic startle response, contextual and auditory cued conditioned fear and hotplate tests.

Heterozygous deleted mice have no common DGS/VCFS phenotypes

As noted above, mice heterozygous for the deletion were healthy and viable, with lifespans comparable with those of the wild-type mice. Tissues from wild-type and heterozygous deleted littermates in both inbred ($n = 10$ wild-type and $n = 11$ heterozygotes between 2 and 36 weeks of age) and mixed ($n = 5$ wild-type and $n = 8$ heterozygotes between 2 and 17 weeks of age) backgrounds were examined histologically for phenotypes displayed by patients with DGS or VCFS. The thymus, parathyroid glands and heart from heterozygous deleted mice were examined microscopically and they all appeared normal (data not shown). We have detected no cardiac abnormality, including no conotruncal defect, normal cardiac papillary muscle (Fig. 4A), tricuspid valve (Fig. 4A and B), normal pulmonary valve (Fig. 4C) and no ventricular septal defect including normal conal septum. In addition, the aortic arch and vessels were normal (Fig. 4D). We used a knock-in allele for *Cripto* (28), a gene expressed in the cardiac outflow tract, to examine embryonic conotruncal development at 10.5 days post-conception. *Cripto* expression in the heterozygous embryos (Fig. 4F and H) was indistinguishable from that in the wild-type (Fig. 4E and G), and cardiac cushion mesenchyme, outflow tract and myocardium appeared normal.

At birth and 10 days of age, serum calcium and phosphate concentrations were normal in heterozygotes and indistinguishable from levels in wild-type littermates (data not shown), demonstrating that the normal appearing parathyroid glands were functional. Flow cytometry analyses of T cell development in the thymus (using CD3, CD4 and CD8

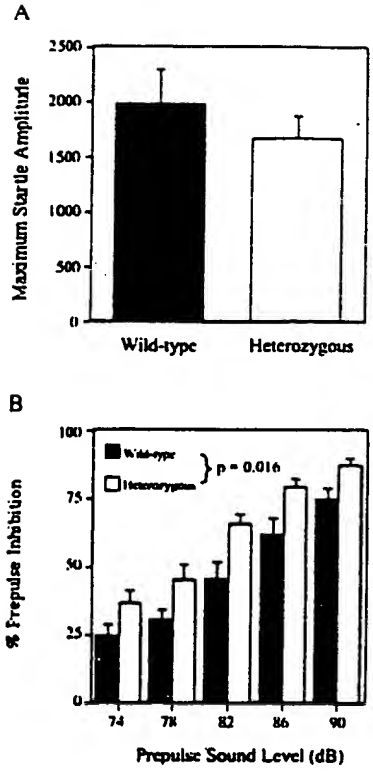

Figure 3. Abnormal PPI of the startle response in heterozygotes. Startle amplitude to the 120 dB stimulus (A) and levels (%) of prepulse inhibition of the acoustic startle response (B) for heterozygous (open bars) and wild-type (closed bars) male mice. Overall, percent prepulse inhibition was significantly higher for heterozygous mice than wild-type mice ($P = 0.016$). There were no significant differences between the mixed (129S6 × Black Swiss) and inbred (129S6) genetic backgrounds. Data are represented as means (± SEM). In addition, we have evaluated the performance of DGDD heterozygous mice in a complete test battery and found no other significant differences that were observed in both genetic backgrounds (see text).

markers) and of T and B cell development in the spleen (using B220 and TCR antibodies) were normal in heterozygous mice and indistinguishable from wild-type (data not shown). Therefore, mice heterozygous for the ~150 kb deletion in the conserved DGCR display no common phenotypes of DGS or VCFS.

DISCUSSION

We have produced mice with a deletion of a portion of the DGCR syntenic region. This deletion encompasses ~150 kb and seven genes, six of which are in the MDGCR. Detailed phenotypic analysis of heterozygous and homozygous deleted mice demonstrated that this region is essential for normal prepulse inhibition and that homozygous deleted mice died early in embryogenesis, soon after implantation. However, heterozygous mice displayed none of the phenotypes commonly found in humans with DGS/VCFS.

The most striking phenotype in the heterozygotes was that they displayed increased PPI of the startle response. Elevated PPI was seen in two different groups of deletion mice and in two different genetic backgrounds, suggesting that this is a robust behavioral effect. In addition, this abnormality is specific, since we observed no other defects in the broad behavioral battery, with the exception of rotarod performance. However, rotarod performance was impaired only in mice of mixed genetic background. In the course of performing 20 or more tests, at the significance level of 0.05, one test would be expected to be abnormal by chance. The reproducibility of elevated PPI in the mutants and the independence of this effect of genetic background and gender gives further confidence in the validity of this finding.

There are at least two mechanisms by which this deletion could result in abnormal sensorimotor gating. One or more genes in the deleted region may have a dosage-sensitive effect on pathways important for PPI. Conversely, the deletion may cause position effects on the expression of genes outside the deleted region that are critical for sensorimotor gating. It is of interest that a gene that maps to the DGCR is proline dehydrogenase. This gene is deleted is some cases of DGS and recently it was shown that mice with a point mutation in this gene display attenuated sensorimotor gating (29). This gene has been mapped in mouse and lies distal to Dgcr6 and, therefore, is outside our deleted region (30). Thus, the relationship between our findings and those for Prdh is unclear at present.

Patients with a number of neuropsychiatric disorders, including schizophrenia, have impaired sensorimotor gating as assessed by PPI (reviewed in ref. 31). Individuals with deletions in the DGCR develop abnormal behavioral neuropsychiatric responses that are remarkably similar to patients with schizophrenia or schizotypal disorder (5,17,32). In addition, several groups have mapped a susceptibility locus for schizophrenia to the DGCR (20–22). We were surprised to find that heterozygous deleted mice displayed increased sensorimotor gating, the opposite abnormality to that seen in patients with schizophrenia. Unfortunately, there are no published reports describing sensorimotor responses of individuals with DGS or VCFS, so at the moment it is unclear whether the abnormalities that we observed in the deleted mice have direct relevance to human 22q11 syndromes.

We have performed a detailed analysis for the common phenotypes of DGS/VCFS and have found no such abnormalities in mice heterozygous for the deletion. These results demonstrate that in the mouse, deletion of this region is not sufficient to reproduce the syndrome. There are several potential reasons for the lack of a DGS/VCFS phenotype in these mice. First, genes within our deleted region may not be involved in the pathogenesis of DGS/VCFS in human. Secondly, we may not have made the appropriate deletion and only a more extensive deletion will result in the loss of sufficient genetic material to produce DGS/VCFS phenotypes. Thirdly, there may be genetic redundancy in the mouse that is not present in the human. Fourthly, neural crest migration, which appears to be a potential mechanism for the pathogenesis of 22q11 deletion syndromes, may be different in the mouse and not involve the same genetic program. This is an unlikely explanation, since it has been possible to model several human neural crest migration mutants in the mouse. Finally, the mouse may not be a good system to model DGS/VCFS phenotype because the genetic organization is different in mouse and human. Comparative mapping between human and mouse genomes has shown that the syntenic region of human 22q11 lies on mouse chromosome 16. Nearly all of the genes identified within the DGCR are linked on mouse chromosome 16. However, gene order is conserved only in blocks

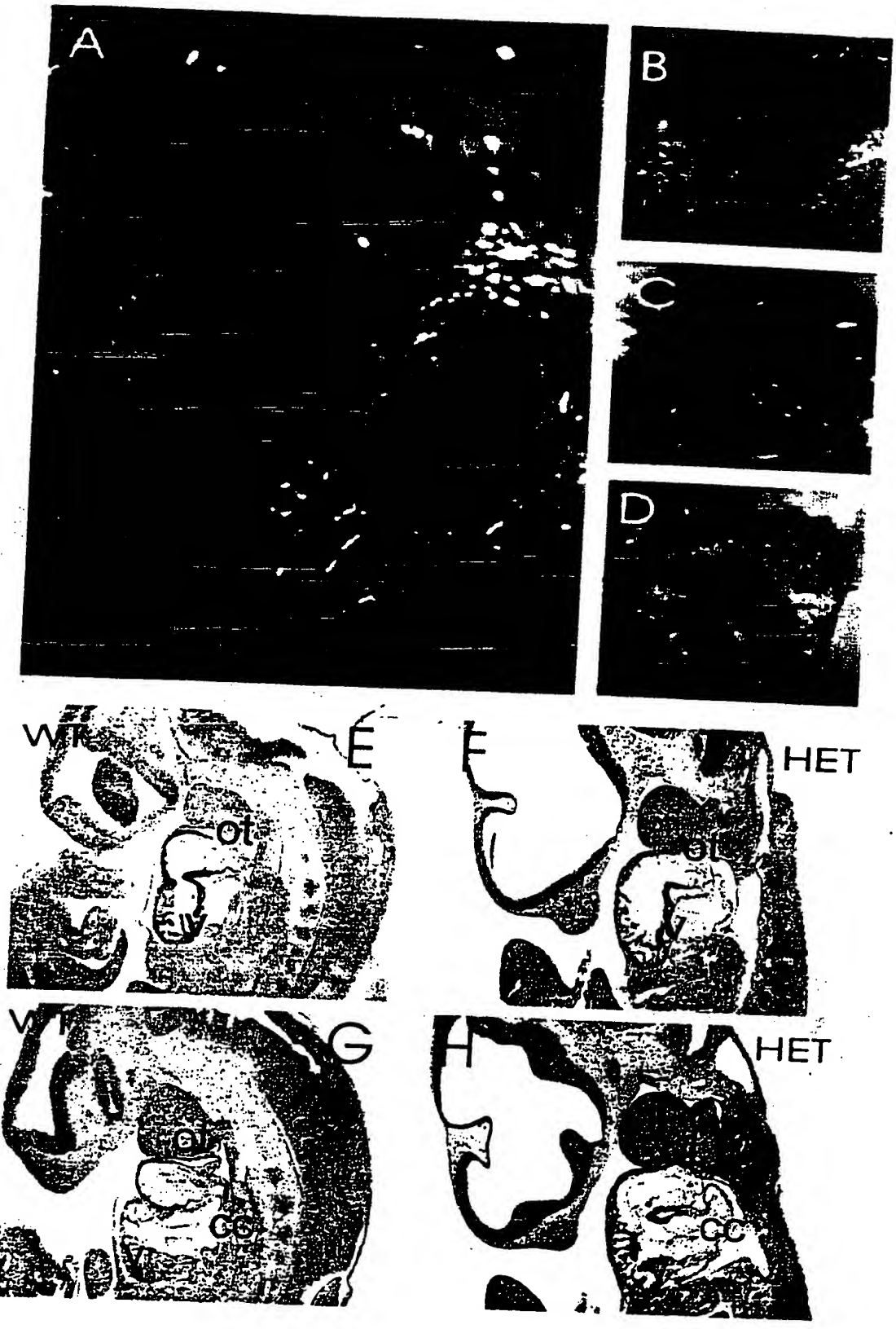

Figure 4. Examination of cardiac morphology and development. Hearts from adult heterozygous mice were isolated, dissected and anatomy observed with a stereomicroscope (10× magnification). The myocardial wall was sectioned and the integrity of (A) papillary muscles (pm), (B) tricuspid valve [tcv, see also (A)], (C) pulmonary valve (pv) and (D) aortic arch (ao) was determined. No significant differences were observed relative to wild-type in six animals of each genotype. The development of the outflow tract was examined by mating the heterozygous mice with a *lacZ* knock-in of *Cripto* (28), a gene expressed in the outflow tract at 10 days post-conception. There were no significant differences in *Cripto* expression or outflow tract morphology between wild-type (E and G) and heterozygous (F and H) littermates.

of this region (30,34–37), so the overall genomic organization is very different. If DGS/VCFS phenotypes are caused by position effects, and not simply deletion of genes, the appropriate positional effects may be difficult to replicate in the mouse.

After our manuscript was accepted for publication, Baldini reported that substantially larger deletions of the DGS/VCFS region resulted in incompletely penetrant defects in the aortic arch (33). In this study a region was deleted from *Es2*, a gene within our deleted region, to *Ufd1l*, which is well distal to *Ctp*. We did not observe any aortic arch defects (Fig. 4), suggesting that the region between *Es2* and *Ctp* does not contain the gene(s) responsible for the defects observed in mice with the larger deletion.

The early post-implantation embryonic lethality observed in homozygous deleted mice demonstrates that a gene or genes present in the deleted region is essential for very early embryonic events. In fact, the effect of these genes may be even earlier than post-implantation, since maternal proteins for genes in the deleted region are likely to be present in early embryos. Several candidate genes in this region may be responsible for this phenotype, although it has been shown from two independent knockout studies that mice which are homozygous for null mutations of *Gscl* are completely viable (10,11). The analysis of phenotypes of mice with inactivation of single genes will be required to determine whether the loss of one or more genes is essential for early post-implantation development.

In summary, our analyses of mice with heterozygous deletion of seven genes in the DGCR does not appear to disrupt development of branchial derivatives in mice nor does it produce common pathological manifestations of DGS and other related 22q11 haploinsufficiency syndromes. However, deletion of this region does result in abnormal sensorimotor gating. Although it appears that haploinsufficiency of these genes is insufficient for pathogenesis of DGS/VCFS, these genes may nevertheless contribute to the process. The analysis of larger deletions will be required to further investigate the role of the DGCR in the mouse.

MATERIALS AND METHODS

Production of mice with a heterozygous deletion within the murine DGCR

Two overlapping BAC clones (36), 399N12 and C3c39 (327L8), from the conserved region at the proximal end of the murine DGCR were used to produce the targeting construct (Fig. 1A). These two BACs were chosen because they displayed a minimal overlap and spanned the largest contiguous region to make a deletion construct. The details of cloning will be presented in another manuscript describing the use of replacement-type targeting to produce 100–200 kb deletions (S. Hirotsune, W. Kimber and A. Wynshaw-Boris, manuscript in preparation). The distal, divergent ends of each of the BACs were subcloned for mapping and to produce the targeting construct. Approximately 15–20 kb of flanking sequence from each end was used in producing the targeting construct (Fig. 1B). The targeting construct was designed to replace ~150 kb of this region with the PGK*neo* selectable marker. From the end cosmid subclones, we isolated the flanking probe used for screening by Southern blot analysis and the internal probe used to confirm the lack of rearrangement of the opposite end of the recombinant, which are shown (Fig. 1B).

TC1 ES cells (38) were transfected with the targeting construct as described (38,39). More than 1000 ES clones were picked and screened by Southern blot hybridization after *Kpn*I digestion, using the flanking probe shown. The wild-type (WT) allele gave a band of ~25 kb, while the deleted (DT) allele gave a band of ~30 kb. In the initial screen, two clones were positive (278 and 773; data not shown). These two clones were expanded and the Southern hybridization experiment using *Kpn*I and the flanking probe was repeated (Fig. 1B). To test for rearrangements of the other arm of the targeting construct, the inside probe shown was used with *Kpn*I (Fig. 1B) and *Eco*RI (data not shown) digestions. No aberrant bands were found, demonstrating that no gross rearrangements had occurred in these clones. PFGE was used to examine targeting in these two clones. Clone DNA was digested with *Not*I, separated by PFGE, transferred to nitrocellulose and hybridized to three separate probes, A, B and C. These probes allowed us to determine the fidelity of targeting across the locus. With probe A, the WT allele was 160 kb, whereas the DT allele was 370 kb, due to the loss of *Not*I sites in the deleted region. With probe B, the DT allele was undetectable, whereas the WT allele was 350 kb. Finally, using probe C, the WT allele was 350 kb, the same size as the WT allele using probe B, whereas the DT allele was 370 kb, the same size as the DT allele using probe A. Finally, FISH was used to determine that the ES clone had in fact deleted the appropriate region. Using probes in the deleted region, both clones displayed loss of the deleted region (data not shown). However, clone 278 was a mixed population of properly deleted and undeleted clones. Both clones were injected into blastocysts as described (38,39) and high grade chimeras were produced. Mating of chimeras made from clone 278 did not result in germline transmission, probably because it was a mixed clone. However, mating of chimeras made from clone 773 resulted in germline transmission and we were able to produce heterozygous lines in both mixed (129S6 × Black Swiss) and inbred (129S6) backgrounds as described (31,40).

All studies were approved by an Animal Care and Use Committee and conform to the NIH guidelines for the use of animals in research.

Behavioral analysis

We used eight wild-type male and 10 heterozygous deleted male mice that were of a mixed genetic background (NIH Black Swiss × 129S6) and six wild-type and eight heterozygous male mice of a pure 129S6 inbred genetic background for behavioral studies. The mice were shipped to Baylor College of Medicine at 2 months of age. Behavioral studies were started 1–2 months later. Female wild-type and heterozygous mice on the mixed genetic background have been evaluated, but since we have not studied female mice from the 129S6 inbred background we did not include the female data in any of the formal analyses.

To increase our confidence that any significant phenotype was reliable, mice were tested in three separate batches on different days. More specifically, the mixed background mice were divided into two batches that were tested on different

days and the 129S6 mice were tested on a separate day from either mixed background batch. More details on the behavioral testing procedures have been published (41).

For the behavioral battery, male wild-type and heterozygous deleted mice from both mixed and inbred genetic backgrounds were evaluated on the following assays in the order presented: (i) a neurological screen to assess simple sensory/motor reflexes and responses; (ii) activity in an open field arena to assess locomotor activity and anxiety; (iii) exploration in a light–dark box to study anxiety-related responses; (iv) an accelerating rotarod test to assess motor coordination and skill learning; (v) acoustic startle and prepulse inhibition of the acoustic startle response to assess sensorimotor gating; (vi) habituation of the acoustic startle response to assess simple non-associative plasticity of a sensorimotor response; (vii) contextual and auditory cued conditioned freezing behavior to assess conditioned fear learning; and (viii) a hotplate test for analgesia-related responses. Each of these assays was performed ~1 week apart, except for the fear conditioning experiments, which were performed 2 weeks after habituation of the acoustic startle response. Spatial learning performance was also evaluated in several mutant and wild-type mice, but several wild-type mice from the mixed genetic background had some difficulties swimming so these data are not included. The nature of the poor swimming performance is unknown.

Prepulse inhibition of the acoustic startle response was measured using the SR-Lab System (San Diego Instruments, San Diego, CA). A test session began by placing a mouse in the Plexiglas cylinder where it was left undisturbed for 5 min. A test session consisted of seven trial types. One trial type was 40 ms, 120 dB sound burst used as the startle stimulus. There were five different acoustic prepulse plus acoustic startle stimulus trials. The prepulse sound was presented 100 ms before the startle stimulus. The 20 ms prepulse sounds were 74, 78, 82, 86 or 90 dB. Finally, there were trials where no stimulus was presented to measure baseline movement in the cylinders. Six blocks of the seven trial types were presented in pseudo-random order such that each trial type was presented once within a block of seven trials. The average intertrial interval was 15 s (range 10–20 s). The startle response was recorded for 65 ms (measuring the response every 1 ms) starting with the onset of the startle stimulus. The background noise level in each chamber was 70 dB. The maximum startle amplitude recorded during the 65 ms sampling window was used as the dependent variable. The following formula was used to calculate percent prepulse inhibition of a startle response: 100 – [(startle response on acoustic prepulse plus startle stimulus trials ÷ startle response alone trials) × 100]. Thus, a high percent prepulse inhibition value indicates good prepulse inhibition, i.e. the subject showed a reduced startle response when a prepulse stimulus was presented compared with when the startle stimulus was presented alone. Conversely, a low percent prepulse inhibition value indicates poor prepulse inhibition, i.e. the startle response was similar with and without the prepulse. Two-way (genotype × genetic background) or three-way (genotype × genetic background × a repeated measure such as time) analyses of variance tests were used to analyze the data. Newman–Keuls and simple effects analysis were used for *post hoc* comparisons of main effects and interactions, respectively.

Histopathological analysis

Tissues were collected and placed in 20 vol of 10% buffered formalin. Fixed tissues were either frozen or embedded in paraffin, sectioned and stained using routine methods (42). Sections were examined and photographed under a light microscope. At least 10 adults and three neonates of each genotype were examined.

Flow cytometry analysis

Thymus and spleen were harvested from wild-type and heterozygote 2-week-old mice by standard methods (43) and mixed with the conjugated antibodies (Pharmingen) anti-CD3–CyChrome™, anti-CD4–PE and anti-CD8–FITC or anti-B220–PE and anti-TCR β-chain–FITC. Appropriate isotypic controls were used to set flow cytometer compensation.

Serum calcium and phosphate measurements

Blood samples were isolated from the aorta, spun for 3 min and the serum separated. Aliquots of ~20 µl of serum were loaded onto VetTest Ca and VetTest Phos slides. Samples were measured with the VetTest 8008 machine.

Blastocyst explant cultures

Forty blastocysts were isolated from females of a heterozygous cross at 3.5 days post-conception, placed in individual wells with Dulbecco's modified Eagle's medium with 10% fetal calf serum and photographed daily for 6 days as described previously (38,40). Blastocyst outgrowths were genotyped by nested PCR. The first PCR reaction used the primer pair Tsk1 Nested-5′ (5′-CGGGATGATAGTGGTCGACTGATA-3′) and Tsk1 Nested-3′ (5′-CAGCAGTGGTTAAGGATCT-CATCG-3′) for the wild-type allele and the primer pair Neo5′ (5′-AGAGGCTATTCGGCTATGACTG-3′) and Neo3′ (5′-TTCGTCCAGATCATCCTGATC-3′) for the deleted allele. Both PCR reactions were 25 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min. The nested PCR used the primer pair Tsk1 Nested2-5′ (5′-TGTGGGTCAGCAGCTTAT-3′) and Tsk1 Nested3-3′ (5′-GTAGATGAGGTCCTTGCA-3′) for the wild-type allele and the primer pair Neo Nested-5′ (5′-TGAATGAACTGCAGGACGAGGCA-3′) and Neo3′ (5′-TTCGTCCAGATCATCCTGATC-3′) for the deleted allele. Both PCR reactions were 25 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min.

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